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AN EVALUATION OF TAXONOMIC BOUNDARIES IN *PLATANTHERA DILATATA* (ORCHIDACEAE) BASED ON MORPHOLOGICAL AND MOLECULAR VARIATION

LISA E. WALLACE
Department of Evolution, Ecology, and Organismal Biology,
The Ohio State University, Columbus, OH 43230
Current Address: Department of Biology, University of South Dakota,
Vermillion, SD 57069
e-mail: lwallace@usd.edu

ABSTRACT. *Platanthera dilatata* (Orchidaceae) is a morphologically variable species encompassing three varieties: *dilatata*, *albiflora*, and *leucostachys*. Spur length and geographic distribution are commonly used to distinguish the varieties, but these characters overlap and are not useful for distinguishing the taxa in all instances. In this study, taxonomic boundaries within *P. dilatata* are reevaluated using variation in 16 morphological traits, six ISSR primers, four RAPD primers, and RFLPs of two chloroplast regions. Morphological, ISSR, and RAPD markers revealed groupings that loosely corresponded to the three recognized varieties, but there was a substantial amount of overlap among the taxa, and no markers alone reliably distinguished the varieties. Some variation was detected in the two chloroplast regions, but it did not correspond well with taxonomy or geography. These data also revealed a surprisingly strong degree of divergence between eastern and western populations of var. *dilatata* at ISSR and RAPD loci, but not in morphological or chloroplast markers. Collectively, these results suggest a relatively recent divergence among the three varieties and among populations of var. *dilatata*. Furthermore, these taxa are likely still in a very active state of evolution due to a combination of geographic isolation and selection by pollinators. Because each of the varieties is generally distinguishable by a set of floral traits and molecular markers, their recognition at the subspecific level is supported. Taxonomic revision within var. *dilatata* is not recommended until morphological and molecular variation is examined in populations throughout its range.

Key Words: *Platanthera dilatata*, Orchidaceae, morphology, ISSR, RAPD, PCR-RFLP, *rpl*16 intron, *trn*T-F

Taxonomic classification of *Platanthera* section *Limnorchis* has been a point of contention among orchidologists for more than a century. An inability to classify species due to extensive intraspecific morphological variation and the apparent presence of interspecific hybrids has resulted in little agreement on the number of or defining characters for species (e.g., Ames 1910; Rydberg 1901; Schrenk 1978; Sheviak 1999). Within section *Limnorchis*, *P. dilatata* (Pursh) Lindl. *ex* Beck is easily distinguished among the many green-flowered forms by having white

flowers. However, examination of this species throughout its range revealed a great deal of variation in floral and vegetative traits. Several authors have noted this variation and named specific or subspecific taxa. For example, Rydberg (1901) recognized nine white-flowered species in *Limnorchis*. By contrast, Ames (1910) and others combined all white-flowered plants of section *Limnorchis* together in one or a few taxa. Luer (1975), in the most recent taxonomic treatment of the white-flowered forms, considered a single species, *P. dilatata*, and three varieties, *dilatata* (Pursh) Lindl. *ex* Beck, *albiflora* (Cham.) Ledeberg, and *leucostachys* (Lindl.) Luer. These taxa are distinguished primarily by geographical distribution and floral structure. The distribution of var. *dilatata* encompasses the ranges of the other varieties, which occur throughout western North America. The nominate variety occurs as far west as Alaska, and as far east as Newfoundland. Its southern boundary is approximately New Mexico in the West, and New England in the East. Populations of var. *dilatata* are not abundant in the midwestern United States and Canada. Variety *albiflora* occurs at higher elevations in the Rocky Mountains and other Pacific ranges, and its distribution extends from Alaska to Colorado. Variety *leucostachys* also occurs in the Rocky Mountains and throughout much of the Pacific Northwest, California, Nevada, and Arizona. No consistent differences in habitat type have been noted, with the possible exception of var. *albiflora* favoring higher elevations (Luer 1975). All three varieties are commonly found in mesic sites that are mid-successional or routinely disturbed, have calcareous soils, and receive full sun. Common habitats include roadside ditches, mountain meadows, stream banks, lake shores, and fens.

The flowers of *Platanthera dilatata* are small (1–2 cm wide) and white, occasionally with greenish sepals and ovaries. Flowers occur in inflorescences containing fewer than 20 flowers to more than 75 flowers. The lips of the flowers are strongly basally dilated in all three varieties. The varieties are most readily distinguished by the length of their floral spur. In var. *dilatata* the spur is approximately equal to the length of the lip, in var. *albiflora* the spur is one quarter to one half as long as the lip, and in var. *leucostachys* the spur is approximately twice as long as the lip. The apparent differences in spur size are thought to directly influence the effectiveness of pollinators servicing the flowers. Large noctuid moths (Kipping 1971) and swallowtail butterflies (L. Wallace, pers. obs.) are confirmed pollinators of var. *leucostachys*, and a skipper and several noctuid moths are confirmed pollinators of var. *dilatata* (Boland 1993). Lastly, viscidium (i.e., the sticky pad at the base of a pollinarium) shape may also be helpful for distinguishing among the

varieties (Sheviak 1999). The utility of this character is investigated in this study.

Despite numerous anecdotal accounts of morphological variation in *Platanthera dilatata*, surprisingly few studies have quantified intraspecific variation in floral or vegetative characters across multiple populations. Studies that have reported on morphological variation (e.g., Boland 1993; Catling and Catling 1997; Reddoch and Reddoch 1997) have only examined var. *dilatata* at small regional scales. Additionally, phylogenetic analyses of section *Limnorchis* based on sequences of nuclear rDNA loci did not reveal much variation among the varieties (Wallace 2002), despite the morphological distinctions that are apparent. Thus, the current taxonomy of the white-flowered forms of section *Limnorchis* is still debatable. In this study, individuals were initially assigned to a group corresponding to the gross morphological features described by Luer (1975). This taxonomic classification is evaluated by comparing variation in additional morphological characters with variation at molecular loci using data from random amplified polymorphic DNA (RAPD) loci, inter-simple sequence repeat (ISSR) loci, and restriction fragment length polymorphisms (RFLP) isolated from the *rpl*16 intron and *trn*T-F intergenic region of the chloroplast genome.

MATERIALS AND METHODS

Morphological variation. Morphological diversity was assessed from 122 individuals in a total of 30 populations (Table 1). Floral morphological measurements were taken on up to five individuals per population. Flowers were chosen from the middle of the inflorescence to minimize potential placement effects on floral morphology. Field-collected flowers were preserved in FAA (45% EtOH, 45% water, 5% glacial acetic acid, and 5% formalin) and morphological measurements were taken from preserved flowers. Voucher specimens for populations are deposited at os. Preserved flowers from all populations remain with the author (complete data set of morphological measurements is available upon request from the author). Twelve quantitative traits were assessed on one flower from each plant included in the survey (Table 2). Floral characters included minimum and maximum lip width, lip length from the tip to the point of attachment to the rest of the flower, spur length from the opening to the tip, width of the anther at the apex and base (i.e., where the viscidia are held), dorsal sepal length and width at the widest point, lateral sepal length and width at the widest point, and lateral petal length and width at the widest point. Viscidium shape and lip shape were also noted

Table 1. Location information for populations of *Platanthera dilatata* vars. *dilatata* from the East (E Dil) or the West (W Dil), *albiflora* (Alb), and *leucostachys* (Leu) included in this study. Populations for which morphological and molecular data were collected are indicated by an asterisk (*). Vouchers deposited at OS are indicated by their collection number.

Variety	Location	Voucher ID
E Dil	Fen, Ontario, Canada*	*L. E. Wallace 233*
E Dil	Fen, Ontario, Canada	*L. E. Wallace 234*
E Dil	Beach bog, Ontario, Canada	*L. E. Wallace 232*
E Dil	Bog, Aroostook Co., ME*	*L. E. Wallace 228*
E Dil	Swamp, Herkimer Co., NY*	*L. E. Wallace 220*
E Dil	Fen, Caledonia Co., VT*	*L. E. Wallace 224*
W Dil	Meadow on Kenai Peninsula, AK*	*J. V. Freudenstein 2635a #13–26*
W Dil	Roadside ditch, Park Co., MT*	*L. E. Wallace 246*
W Dil	Seeping roadside, Ravalli Co., MT*	*L. E. Wallace 237*
W Dil	Stream bank, Sublette Co., WY*	*L. E. Wallace 258*
W Dil	Around a lake, Sublette Co., WY*	*L. E. Wallace 259*
Alb	Stream bank, Beaverhead Co., MT*	*L. E. Wallace 241*
Alb	Wet meadow, Ravalli Co., MT*	*L. E. Wallace 238*
Alb	Stream bank, Fremont Co., WY*	*L. E. Wallace 256*
Alb	Wet meadow, Teton Co., WY*	*L. E. Wallace 252*
Alb	Stream bank, Teton Co., WY*	*L. E. Wallace 261*
Alb	Stream bank, Teton Co., WY*	*L. E. Wallace 263*
Leu	Roadside ditch, Idaho Co., ID	*L. E. Wallace 204*
Leu	Roadside ditch, Idaho Co., ID	*L. E. Wallace 205*
Leu	Roadside ditch, Flathead Co., MT*	*L. E. Wallace 214*
Leu	Roadside ditch, Flathead Co., MT	*L. E. Wallace 217*
Leu	Wet forest, Flathead Co., MT	*L. E. Wallace 208*
Leu	Roadside ditch, Missoula Co., MT*	*L. E. Wallace 213*
Leu	Stream bank, Lake Co., MT	*L. E. Wallace 212*
Leu	Roadside ditch, Lake Co., MT	*L. E. Wallace 216*
Leu	Around a lake, Ravalli Co., MT*	*L. E. Wallace 236*
Leu	Roadside ditch, Klamath Co., OR*	*L. E. Wallace 231*
Leu	Wet meadow, Wallowa Co., OR*	*L. E. Wallace 201*
Leu	Roadside ditch, Wallowa Co., OR	*L. E. Wallace 206*
Leu	Roadside ditch, Wallowa Co., OR	*L. E. Wallace 207*

for all flowers. While collecting samples, some differences in the sizes of plants and flowers were noticed between eastern and western populations of var. *dilatata*. Thus, in the statistical analysis, samples of var. *dilatata* were divided into eastern (east of the Mississippi River) and western (west of the Mississippi River) groups for comparison across the range of this taxon. Kruskal-Wallis non-parametric tests (Zar 1996) were used to detect global significance. Dunn's multiple comparisons tests (Zar 1996)

Table 2. Mean (± 1 SD) of 12 quantitative morphological characters (in mm) measured on *Platanthera dilatata* var. *dilatata* from the East (E Dil) or the West (W Dil), var. *albiflora* (Alb), and var. *leucostachys* (Leu). Dunn's multiple comparisons tests were used to compare mean differences across the groups designated. A significant difference ($P < 0.05$) for a trait between groupings is indicated by unlike letters. Sample sizes (n) are indicated for each grouping.

	Mean Size (mm)			
Floral Trait	E Dil (n = 30)	W Dil (n = 25)	Alb (n = 30)	Leu (n = 37)
Lip max width	2.07 ± 0.420^{ab}	2.42 ± 0.476^{b}	1.98 ± 0.301^{a}	2.21 ± 0.438^{ab}
Lip min width	1.07 ± 0.215^{a}	1.09 ± 0.245^{a}	0.98 ± 0.202^{a}	1.03 ± 0.270^{a}
Lip length	5.20 ± 0.890^{ab}	4.18 ± 0.924^{ac}	4.46 ± 0.551^{c}	5.94 ± 1.112^{b}
Spur length	5.15 ± 0.708^{a}	4.40 ± 0.913^{a}	3.11 ± 0.569^{c}	8.79 ± 1.794^{b}
Dorsal sepal length	4.41 ± 0.783^{a}	4.01 ± 0.542^{ab}	3.84 ± 0.459^{b}	5.07 ± 0.927^{c}
Dorsal sepal width	2.50 ± 0.472^{a}	1.92 ± 0.716^{b}	1.36 ± 0.249^{c}	1.68 ± 0.622^{bc}
Lateral sepal length	5.12 ± 1.035^{a}	4.53 ± 0.612^{ac}	4.35 ± 0.605^{c}	5.97 ± 1.220^{b}
Lateral sepal width	2.27 ± 0.411^{a}	2.28 ± 0.498^{a}	1.80 ± 0.418^{b}	2.15 ± 0.731^{ab}
Lateral petal length	4.34 ± 0.961^{ab}	3.69 ± 0.741^{bc}	3.43 ± 0.614^{c}	5.08 ± 1.240^{a}
Lateral petal width	2.24 ± 0.493^{a}	2.63 ± 0.404^{b}	2.35 ± 0.426^{ab}	2.67 ± 0.621^{b}
Anther apical width	0.94 ± 0.189^{a}	1.16 ± 0.242^{b}	1.04 ± 0.247^{ab}	1.25 ± 0.252^{b}
Anther basal width	1.11 ± 0.112^{a}	1.16 ± 0.136^{a}	1.13 ± 0.162^{a}	1.20 ± 0.248^{a}

were subsequently used to determine which groups differed significantly. Statistical tests were performed using SPSS, version 10 (SPSS, Chicago, IL).

Molecular variation. Genotypes of 99 individuals of *Platanthera dilatata*, representing four populations of eastern var. *dilatata*, five populations of western var. *dilatata*, six populations of var. *albiflora*, and five populations of var. *leucostachys*, were identified using ISSR, RAPD, and chloroplast RFLP markers (Table 1). Not all populations included in the morphological analysis were analyzed with molecular markers, but all of the geographic regions are represented in the molecular data sets. Leaves from five individuals per population were sampled.

Leaf samples were kept on ice in the field and stored at –80°C until DNA was extracted. Total genomic DNA was extracted from approximately 0.06 gm of leaf material using a modification of the CTAB method

Table 3. Primer sequence, annealing temperature, and number of bands scored for primers used in this study. Superscripts following the names of RAPD primers are names assigned by Operon Technologies. Taxonomic designations follow those in Table 1.

			Number of Bands Scored			
Primer Name	Sequence 5′ to 3′	Temp.	E Dil (n = 20)	W Dil (n = 25)	Alb (n = 29)	Leu (n = 25)
ISSR-1	$(TC)_6RG$	46°C	15	19	23	23
ISSR-2	$(TC)_6RC$	45°C	7	13	9	18
ISSR-3	$(CA)_7YG$	47°C	10	16	17	15
ISSR-4	$(AC)_7RG$	47°C	16	18	26	24
ISSR-5	$(CTC)_7RC$	45°C	19	20	25	18
ISSR-6	$(CT)_8RG$	48°C	15	16	26	21
RAPD-1^{BE-19}	AGGCCAACAG	36°C	15	20	24	26
RAPD-2^{C-16}	CACACTCCAG	36°C	6	7	7	5
RAPD-3^{X-03}	TGGCGCAGTG	36°C	10	10	11	10
RAPD-4^{X-13}	ACGGGAGCAA	36°C	15	20	21	21
Total			128	159	189	181
Monomorphic			10	6	6	6
Unique			7	9	17	18

(Doyle and Doyle 1987). Template DNA was quantified by gel, and DNA from at least one individual per population was tested in dilutions to determine an appropriate amount for consistent results. In an initial survey, a subset of individuals of each of the varieties was examined for variation with 22 ISSR primers and 62 RAPD primers. Six ISSR primers and four RAPD primers were chosen for the larger survey because they produced repeatable patterns of variation (Table 3). For ISSR primers, each 25 μl reaction contained 1× PCR buffer (20 mM Tris-HCl and 50 mM KCl; Invitrogen, Carlsbad, CA), 200 μM of each dNTP (Invitrogen), 1 mM $MgCl_2$, 0.4 μM primer (0.8 μM for ISSR-1), 0.5 units of *Taq* DNA polymerase (Invitrogen), and 0.3 μl template DNA. ISSR reactions were subjected to the following thermocycler program: 94°C for 2.5 min. (1 cycle); 94°C for 40 sec., 45°–48°C (depending on the primer; Table 3) for 45 sec., 72°C for 1.5 min. (35 cycles); 94°C for 45 sec., 45°–48°C (depending on the primer; Table 3) for 45 sec., 72°C for 7 min. (1 cycle); soak indefinitely at 4°C. For RAPD primers, each 25 μl reaction contained 1× PCR buffer (20 mM Tris-HCl and 50 mM KCl; Invitrogen), 200 μM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 5 pmoles of primer, 0.5 units of *Taq* DNA polymerase (Invitrogen), and 0.4 μl template DNA. RAPD reactions were amplified according to the following program: 94°C for 2 min. (1 cycle); 94°C for 1 min., 36°C for 1 min., 72°C for 2 min. (35

cycles); 72°C for 7 min. (1 cycle); soak indefinitely at 4°C. A negative control, including all ingredients except template DNA, was included with each set of reactions to detect contamination. The total product was separated on 1.2% TAE agarose gels, stained with ethidium bromide, and visualized with UV light. Images of gels were captured digitally for later analysis. Duplicate reactions and gels were run for all primers and individuals. Non-replicated bands were eliminated from the data set. Band homology was based on similarity of molecular weight and occasionally band intensity. A 1 kb-plus DNA ladder (Invitrogen) was run on each gel as a size standard. Additionally, bands suspected of being similar in size across gels were compared by reamplifying individuals and running them side-by-side on a gel. Bands were scored as present or absent.

Band frequency was determined for each taxonomic group as the number of individuals containing a band relative to the number of individuals surveyed. The data set was examined for the presence of high-frequency, group-specific bands. The data matrix was also examined for fixed genotypes within or between populations. The matrix of bands was subjected to a multivariate analysis using principal coordinates analysis (PCoA) in NTSYS-pc (Rohlf 1998) based on the similarity coefficient of Nei and Li (1979). Mean intravarietal and intervarietal genetic similarity was determined from all inter-individual comparisons using the similarity coefficient of Nei and Li (1979).

Restriction fragment length polymorphisms were also used to elucidate patterns of relatedness and seed dispersal among the varieties. The chloroplast genome is assumed to be passed to offspring maternally as has been demonstrated for other orchids (Chang et al. 2000). Two non-coding regions of the chloroplast genome, the *rpl16* intron and *trn*T-F intergenic region, were amplified by PCR and cut with four restriction enzymes. The *rpl16* intron was amplified using primers F71 (Jordan et al. 1996) and R622 (Les et al. 2002). The *trn*T-F intergenic region was amplified using primers "a" and "f" from Taberlet et al. (1991). Each 25 µl reaction contained 1× PCR buffer (20 mM Tris-HCl and 50 mM KCl; Invitrogen), 200 µM of each dNTP (Invitrogen), 3 mM $MgCl_2$, 0.24 µM of each primer for *rpl16* (0.4 µM of each primer for *trn*T-F), 0.5 units of *Taq* DNA polymerase (Invitrogen), and 1.0 µl template DNA. Amplified products were amplified under the following conditions: 94°C for 5 min. (1 cycle); 94°C for 1 min., 53°C for 1 min., 72°C for 2 min. (35 cycles); a final extension at 72°C for 5 min. (1 cycle). Products were verified on 1% TAE agarose gels and subsequently cleaned by precipitating them with an equal volume of PEG:NaCl (20%:2.5 M). Four µl of the cleaned product were digested

with two units of restriction enzyme for 24 hr. according to the manufacturer's instructions. The *rpl16* intron was digested with *Eco*RV (Invitrogen) while *trn*T-F was digested with *Bst*NI, *Dra*I, and *Mse*I (New England BioLabs, Beverly, MA). Separate reactions were set up for each restriction enzyme. Digested products of *Eco*RV were separated on 1.2% agarose TBE gels; the products of *Bst*NI and *Dra*I were separated on 2% agarose TBE gels, and the products of *Mse*I were separated on 2% NuSieve agarose (BioWhittaker Molecular Applications, Rockland, ME) TBE gels. A 1 kb-plus DNA ladder (Invitrogen) was run on each gel as a size standard. Gels were stained with ethidium bromide and visualized under UV light. Individuals suspected of having similar banding patterns were redigested and run side-by-side on a gel. Bands of similar mobility on a gel were assumed to be homologous and to have or lack a restriction site in common. The number of distinct chloroplast haplotypes was determined, and populations were examined for shared haplotypes.

RESULTS

Morphological variation. The varieties of *Platanthera dilatata* are similar in that they have white flowers with strongly dilated lips and pointed stigmas. The three varieties were found to differ, however, in flower size, spur length, and viscidium shape (Table 2). The variety *leucostachys* generally had larger flowers than either of the other two varieties. The spur was approximately one and a half times longer than the lip in var. *leucostachys* (spur:lip length ratio = 1.48), was shorter than the lip in var. *albiflora* (spur:lip length ratio = 0.70), and was approximately the same length as the lip in var. *dilatata* (spur:lip length ratio = 0.99 and 1.05, respectively for eastern and western populations). Although some differences were detected between eastern and western samples of var. *dilatata*, few of these differences were significant. Western samples of var. *dilatata* did have smaller flowers with shorter lips and spurs, more strongly dilated lips, and wider anther apices than eastern samples (Table 2). The viscidia of varieties *dilatata* and *leucostachys* were square-oblong, but differed in size, with var. *dilatata* having shorter viscidia. The viscidia of var. *albiflora* were oblong to lanceolate in shape and generally smaller than those of the other two varieties. Different floral fragrances were also detected among the varieties, but were not consistent within a variety (L. Wallace, pers. obs.). A clove-like scent was characteristic of eastern populations of var. *dilatata* while western populations of this variety exhibited a much sweeter fragrance. A clove-like scent was also

Table 4. Number of bands shared between groups (below the diagonal) and shared exclusively between two groups (above the diagonal). Taxonomic designations follow those in Table 1.

	E Dil	W Dil	Alb	Leu
E Dil	***	4	4	2
W Dil	105	***	7	6
Alb	112	138	***	21
Leu	104	130	152	***

apparent in some populations of var. *leucostachys*. Populations of var. *albiflora* resembled western populations of var. *dilatata* in floral scent.

Molecular variation. From the six ISSR primers and four RAPD primers used, a total of 237 bands was scored among all surveyed individuals. The ISSR primers yielded twice as many bands overall (158) and a greater mean number of usable bands per primer (mean = 26.33 bands) compared to RAPD primers (mean = 19.75 bands). If all bands or just ISSR bands were considered, every individual could be identified by a unique multilocus phenotype. Generally, multilocus phenotypes were more similar within populations than between populations. Most bands were polymorphic and found in fewer than half of the individuals surveyed for each group. A small number of monomorphic bands (i.e., in every individual surveyed) was found in each of the varieties (Table 3), but none of these bands was unique to a single taxonomic group. A total of 51 unique bands was identified, but most occurred infrequently. No unique band occurred with a frequency of greater than 10%. Nearly half of the bands identified were shared by at least two groups, with *Platanthera dilatata* vars. *albiflora* and *leucostachys* having the greatest number of bands in common (152; Table 4). Eastern var. *dilatata* populations shared fewer than 50% of all identified bands with var. *dilatata* in the West or with vars. *albiflora* or *leucostachys*. Several bands were shared exclusively between two groups. The vars. *albiflora* and *leucostachys* shared 21 bands that were absent in var. *dilatata* (Table 4). Interestingly, 10 bands were shared between eastern var. *dilatata* and a taxon from the West.

Indices of genetic similarity were consistently higher within groups than among groups and ranged from 0.494 in *Platanthera dilatata* var. *albiflora* to 0.650 in eastern var. *dilatata* (Table 5). Considering intergroup comparisons, individuals of var. *dilatata* from eastern and western populations exhibited the highest similarity (0.483) while the greatest dissimilarity occurred between eastern var. *dilatata* and var. *albiflora* (0.409) or var. *leucostachys* (0.415).

Table 5. Mean intragroup (on the diagonal) and intergroup (below the diagonal) genetic similarity based on banding patterns at RAPD and ISSR loci. Mean similarities are based on comparisons of Nei and Li's (1979) similarity coefficient among all interindividual comparisons. Taxonomic designations follow those in Table 1.

	E Dil	W Dil	Alb	Leu
E Dil	0.650			
W Dil	0.483	0.570		
Alb	0.409	0.459	0.494	
Leu	0.415	0.432	0.461	0.533

Even though none of the varieties could be identified by a suite of taxon-specific bands, these data collectively suggest a degree of distinction that is also apparent in the morphological data set. The similarity of banding patterns within groups and the different band frequencies among groups are responsible for the patterns depicted in the PCoA of genetic similarities (Figure 1). Samples of *Platanthera dilatata* var. *dilatata* from eastern North America are clearly different from western var. *dilatata*, var. *albiflora*, and var. *leucostachys*, while samples of the three varieties from western North America overlap to a greater degree. Most individuals of var. *albiflora* appear extremely similar to individuals of var. *leucostachys* in the PCoA plot. The first two axes account for 19.24% of the total variation observed in ISSR and RAPD markers.

Variation in chloroplast RFLP patterns was found within varieties and within populations. Digestion of *rpl16* and *trn*T-F allowed the resolution of 12 distinct chloroplast haplotypes. Fragments of two different sizes were identified in amplifications of the *trn*T-F region. Most individuals contained a fragment of approximately 2.2 kb; a smaller fragment of approximately 1.7 kb was found only in *Platanthera dilatata* vars. *dilatata* and *albiflora*. The remaining variation occurred at restriction sites. There was little correspondence between haplotype and varietal status or geographic origin. All of the eastern samples of *P. dilatata* had an identical haplotype which was shared with a population from Alaska as well as with individuals of vars. *albiflora* and *leucostachys* from the western United States. Multiple haplotypes were found in 10 populations of all three varieties.

DISCUSSION

Variation among the varieties. *Platanthera dilatata* is one of the most morphologically variable species in section *Limnorchis*. Unlike

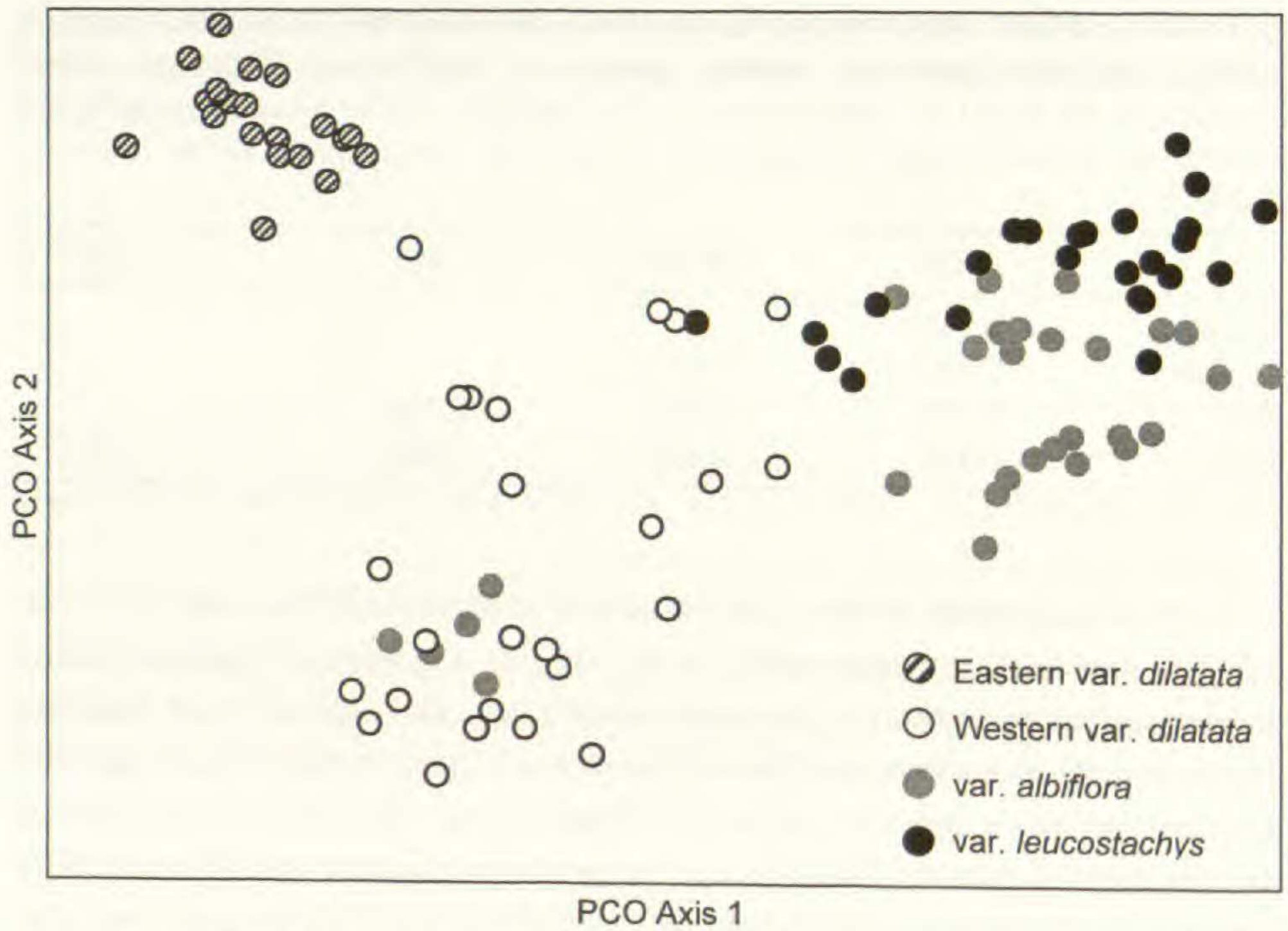

Figure 1. Plot of the first two axes in a principal coordinates analysis based on the similarity coefficient of Nei and Li (1979) derived from ISSR and RAPD banding patterns in each individual of *Platanthera dilatata* surveyed. The first two axes account for 19.24% of the total variation observed.

geographical variants discussed in other species of section *Limnorchis*, many authors agree that variants of *P. dilatata* are generally recognizable in the field. In this study sufficient levels and similar patterns of variation in morphological and molecular ISSR and RAPD markers support Luer's (1975) recognition of three taxa at the rank of variety. Flower size, spur length in relation to lip length, and viscidium shape can be used to distinguish the varieties in most populations. The var. *leucostachys* was found to be the most distinctive, with a spur that was nearly one and a half times longer than the lip (Table 2). Additionally, the flowers of var. *leucostachys* were generally larger, with larger viscidia than in either of the other varieties. Although more similar in overall appearance, vars. *dilatata* and *albiflora* could also be distinguished by spur length and flower size in most populations. The var. *albiflora* displayed very short spurs but slightly larger flowers than var. *dilatata* in the West. Because we found considerable overlap in the sizes of floral features, some individuals with intermediate morphologies will necessarily be difficult to classify. These individuals may be intervarietal hybrids or extremes of one variety or the other; both are earmarks of an actively evolving species.

Variation in floral characters is often an indication of selection by pollinators for traits that increase the chances of successful pollination. Similarly, divergence in floral traits in *Platanthera dilatata* may have been driven by pollinator behavior if the primary suite of pollinators differed across microhabitats. While vars. *dilatata* and *leucostachys* are not specific for a single pollinator species, successful pollination occurs only by lepidopteran insects, and different types of moths and butterflies are confirmed pollinators of these varieties (Boland 1993; Kipping 1971). If primary pollinators differed across microhabitats and populations, divergence in floral traits such as spur length and viscidium shape could occur rather quickly as a result of directional selection by pollinators. Subsequently, new variant populations could spread through long distance seed dispersal (Dodson and Gillespie 1967; Dressler 1993). Although more studies are needed to truly evaluate the strength of selection by pollinators on morphological divergence in *P. dilatata*, there is precedence in other *Platanthera* species. In *P. mandarinorum* (Inoue 1983) and *P. ciliaris* (Robertson and Wyatt 1990), intraspecific variation in floral traits strongly correlates with variation in morphological features of the local pollinator fauna.

Given that at least three morphological variants of *Platanthera dilatata* have been found to be distinct enough to be considered separate taxa, then they are expected to have unique evolutionary histories as well, and patterns of divergence should be traceable with the appropriate molecular markers. However, neither sequence data of nuclear rDNA loci (Wallace 2002) nor the data sets presented in this study indicate with certainty the direction of divergence among the three varieties. No high-frequency variety-specific bands were found, and there is little evidence to suggest that one variety contains a subset of variation found in any other variety. Additionally, many chloroplast haplotypes are shared among the varieties, and no variety exhibits a unique and abundant haplotype. Although principal coordinates analysis suggests a closer relationship between vars. *albiflora* and *leucostachys* than either variety to var. *dilatata*, this result could be interpreted as an indication of derivation from similar genotypes within var. *dilatata* or perhaps that all three varieties evolved independently from a similar ancestral taxon. The low degree of divergence among the varieties, indicated by a lack of fixed or high-frequency molecular markers, suggests that these taxa may have only recently started to diverge. It is expected that geographic isolation and the accompanying effects of genetic drift as well as selection by pollinators were important historical factors promoting divergence in this species.

Geographic variation in var. *dilatata*. The strong differences in ISSR and RAPD banding patterns found between populations of *Platanthera dilatata* var. *dilatata* from eastern and western North America are somewhat surprising, given the lack of significant variation in most morphological traits (Table 2). Because many of the loci amplified by ISSR and RAPD primers are thought to be in non-coding regions, they are expected to evolve rapidly (Wolfe and Liston 1998). It is the differences in the rapidly evolving ISSR and RAPD markers, and a lack of variation in more slowly evolving morphological and chloroplast markers that suggest that divergence of var. *dilatata* populations has also occurred recently. How such a divergence could arise in this taxon is not entirely clear from these data, but several hypotheses are consistent with the data at hand. First, although populations are continuous from the east coast to the west coast, their occurrence may be limited in midwestern North America due to a lack of suitable habitat. Infrequent, widely separated populations may create an effective barrier that prevents substantial gene flow between eastern and western populations. Additionally, differing pollinator faunas across the distribution of plant populations may create a barrier to pollen-mediated gene flow. Alternatively, eastern populations, which exist entirely within the last glacial maximum, may have recolonized the area from different refugia than those from which western populations are derived.

The data presented in this study offer little in the way of differentiating among hypotheses to explain the factors promoting divergence in *Platanthera dilatata*. They have, however, brought to light an interesting example of an actively evolving group and a case of cryptic divergence in var. *dilatata* that was not detectable with morphological or chloroplast markers. Further studies aimed at understanding the distinctness of populations of var. *dilatata* will require sampling populations in the middle part of the range of var. *dilatata*. These populations serve as a bridge, albeit not necessarily functional, between the East and the West. Additional fine-scale molecular markers and their analysis in a genealogical framework may also be helpful for evaluating the taxonomic distinctness of eastern and western var. *dilatata* as well as elucidating factors important for the morphological and molecular distinctness at the varietal level.

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